CHARACTERIZATION OF THE MECHANISM OF THE IMMUNOSUPPRESSIVE ACTIVITY OF INTERLEUKIN 10 ON HUMAN $CD4^+$ T CELLS

By

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TABLE OF CONTENTS

ACKNOWLEDGMENTS ii

LIST OF TABLES vi

LIST OF FIGURES vii

ABSTRACT viii

CHAPTERS

1 INTRODUCTION 1

Cytokines and the Immune System 1
Discovery and Physical Properties of IL-10 2
Biological Activities of IL-10 3
Superantigens 6
IL-10 in Health and Disease 10
Mammalian Cell Cycle 11
T-Cell Receptor Signaling 14
MAP Kinase Pathway 16

2 MATERIALS AND METHODS 18

Isolation of Peripheral Blood Mononuclear Cells and Cell Culture 18
Mixed Lymphocyte Reaction and Proliferation Assay 18
Flow Cytometry 20
Immunoblotting 22
Interleukin 2 assay 23
Extracellular Signal Regulated Kinase Assay 23
Raf-1 Kinase Cascade Assay 24

3 RESULTS 26

Suppression of the Mixed Lymphocyte Reaction 26
Inhibition of SEB-Induced Proliferation of PBMCs. 29
Inhibition of Cell Cycle Progression in SEB-Stimulated $CD4^+$ T cells 29

Time Course of Inhibition of Cell Cycle Progression 31
Maintenance of Tumor Suppressor Protein $p27^{Kip1}$ Levels 34
Inhibition of Cyclin Upregulation 36
IL-10 Inhibits IL-2 Production 36
IL-2 Reverses IL-10 Effects on Cell Cycle Progression 38
IL-2 Partial Reversal of IL-10 Effects on Cell Cycle Proteins 42
Reduced Levels of IL-2 Transcription Regulators c-fos and c-jun 44
IL-10 Directly Blocks Proliferation of $CD4^+$ T Cells 46
Suppression of Extracellular Signal-Regulated Kinase Activity 48
Suppression of Raf-1 Activity 51

4 DISCUSSION 54

REFERENCES 59

BIOGRAPHICAL SKETCH 66

LIST OF TABLES

Table		page
1	Biological Properties of IL-10	5
2.	IL-10 Suppresses Stimulation of $CD4^+$ T Cells	30
3	IL-10 Dose Response	32
4	Time course of IL-10 Addition	33
5	IL-10 Suppresses IL-2 Production	37
6	Early IL-10 Suppression of IL-2 Production	39
7	Addition of IL-2 Reverses IL-10 Immunosuppression No IL-10 Pretreatment	40
8	Addition of IL-2 Reverses IL-10 Immunosuppression 2 Day IL-10 Pretreatment	41

LIST OF FIGURES

	Figure	page
1	Superantigen Binding	8
2	Superantigen Binding in the Absence of MHC Class II	9
3	Mammalian Cell Cycle	12
4	MAP Kinase Pathway	17
5	IL-10 Suppresses the Mixed Lymphocyte Reaction	27
6	IL-10 Suppresses Superantigen Induced Stimulation	29
7	IL-10 Inhibits Superantigen Induced Changes in Cell Cycle Proteins	35
8	Addition of IL-2 Partially Reverses Changes in Cell Cycle Proteins	43
9	IL-10 Reduces Induction of c-fos and c-jun	45
10	IL-10 Directly Blocks Proliferation of $CD4^+$ T Cells	47
11	IL-10 Suppresses Activation of Erk in SEB-Stimulated Cells	49
12	IL-10 Suppresses Activation of Erk in PMA/ionomycin-Stimulated Cells	50
13	IL-10 Suppresses Activation of Raf in SEB-Stimulated Cells	53

Abstract of Dissertation Presented to the Graduate School
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CHARACTERIZATION OF THE MECHANISM OF THE IMMUNOSUPPRESSIVE ACTIVITY OF INTERLEUKIN-10 ON HUMAN $CD4^+$ T CELLS

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Interleukins are proteins produced by cells of the immune system which help regulate the relative state of activation of the immune system Interleukin-10 (IL-10) is a multifunctional cytokine which has immunostimulatory or immunosuppressive properties depending on the target cell type I first investigated the antiproliferative effects of IL-10 in a mixed lymphocyte reaction (MLR) in which peripheral blood mononuclear cells (PBMC) from two separate donors are cultured together The resulting proliferative response was consistently suppressed in the presence of IL-10 There were, however, inconsistencies in both the intensity of the immune response and the degree of immunosuppression caused by donor variation.

To further study IL-10's immunosuppressive effects, I analyzed the effects of IL-10 on the entry of quiescent $CD4^+$ T cells into the cell cycle upon stimulation with the

superantigen staphylococcal enterotoxin B (SEB) IL-10 arrested cells at G_0/G_1. IL-10 treatment prevented the downregulation of $p27^{Kip1}$, an inhibitory protein that controls progression out of the G_0 phase of the cell cycle. IL-10 also prevented the upregulation of the G_1 cyclins D2 and D3, proteins necessary for entry and progression through the G_1 phase of the cell cycle. Associated with the inhibition of the cell cycle, IL-10 suppressed SEB induction of interleukin-2 (IL-2). Addition of exogenous IL-2 to IL-10-treated cells significantly reversed the antiproliferative effect of IL-10 as well as IL-10's effects on the early G_1 proteins $p27^{Kip1}$ and cyclin D2. Although this reversal by IL-2 was pronounced, it was not complete, suggesting that IL-10 may have some effects not directly related to the suppression of IL-2 production. Cell separation experiments suggest that IL-10 can effect purified $CD4^+$ T cells directly, providing functional evidence for the presence of IL-10 receptors on $CD4^+$ T cells. IL-10 also inhibited expression of IL-2 transcriptional regulators c-fos and c-jun, which also inhibit other cell functions. These studies also show that the mechanism of IL-10 regulation of quiescent $CD4^+$ T cell activation is mainly by blocking induction of IL-2 that is critical to downregulation of $p27^{Kip1}$ and upregulation of D cyclins in T-cell activation and entry into the cell cycle.

Finally, my studies show that this blockage of IL-2 induction is directly related to the inhibitory activity of IL-10 on the proteins of the mitogen-activated protein (MAP) kinase pathway, which normally lead to IL-2 induction in activated $CD4^+$ T cells.

CHAPTER 1
INTRODUCTION

Cytokines and the Immune System

Cytokines are small soluble proteins produced mainly by the cells of the immune system. These proteins act primarily to regulate the activation growth and differentiation of various lymphocyte populations. Cytokines may have varying effects on the cells of the immune system ranging from stimulating an immune response, to suppressing an immune response, to inducing the differentiation of immune system cells. These effects are determined by which cytokines are acting and which target cells they are acting upon.

Cytokines are not stored in immune cells as pre-formed proteins and must be synthesized when needed. Cytokine synthesis may be induced in a number of ways including binding of extracellular cytokines to cell surface receptors. Cytokines often influence the synthesis of other cytokines, leading to cascades in which subsequent cytokines may mediate the biological actions of the first cytokine. This ability of one cytokine to enhance or suppress the production of others may provide important positive or negative regulatory mechanisms for immune system responses.

Cytokines often influence the biological activity of other cytokines (Longyal 1982). This can occur either by acting in an antagonistic fashion, producing an additive

effect or by acting synergistically thereby producing an effect greater than the additive effect of the cytokines alone. Cytokines act by binding to surface receptors on target cells. Expression of these receptors may also be regulated by cytokines. It has been shown that T cells express cytokines which can regulate the expression of the IL-2 receptor (Johnson and Farrar, 1983). Expression of a cytokine receptor may be regulated by that same cytokine or by other cytokines.

Discovery and Physical Properties of IL-10

Observations of T helper cell behavior led to the characterization of groups into which most T helper cells could be placed with the 2 major groupings being designated T_H1 and T_H2 (Mosmann et al., 1986; Del Prete et al., 1991). The T_H1 designation applied to those T helper cells which were mainly involved in cell-mediated or delayed type hypersensitivity (DTH) reactions while the T_H2 designation applied to those T helper cells involved in antibody responses. Further observation revealed that these two T helper cell groups were for the most part mutually exclusive; a strong T_H1 response would inhibit a T_H2 response and vice-versa. The type of immune response dominating was determined through the effects of secreted cytokines. The cytokines produced by T_H1 cells, mainly interferon gamma (IFN-γ) and IL-2, promoted the DTH type immune response while suppressing an antibody response. On the other hand, T_H2 cytokines, mainly IL-4 and IL-10, promoted an antibody response while inhibiting a DTH response. IL-10 was first characterized in 1989 by its ability to inhibit production of

some T_H1 cytokines such as IFN γ and IL-2 (Fiorentino et al., 1989). For this reason, it was originally termed cytokine synthesis inhibitory factor.

Human IL-10 is a protein consisting of 160 amino acids with a molecular weight of 18,500 Daltons. In its active form, IL-10 is a non-covalently linked homodimer with predominantly alpha-helical structure. Interestingly, the structure of IL-10 is quite similar to that of IFN-γ, one of the T_H1 cytokines it inhibits (Walter and Nagabhushan, 1995). Human and murine IL-10 share 74% protein sequence homology and human IL-10 is active on both cell types while murine IL-10 is only active on murine cells. IL-10 is an unusually acid labile cytokine, with activity rapidly lost below a pH of 5.5.

Biological Activities of IL-10

IL-10 has been shown to have both immunostimulatory and immunosuppressive properties, depending upon the target cell type (Table 1). IL-10 exerts immunostimulatory properties on B cells and mast cells and is a growth factor for immature thymocytes (Mosmann, 1994). In B cells, IL-10 has been shown to enhance MHC class II expression and increase B cell viability (Go et al., 1990), enhance B cell proliferation (Rousset et al., 1992) and antibody secretion (Pecanha et al., 1992). In T cells IL-10 enhances cytotoxic T cell development (Chen and Zlotnick, 1991) and is a stimulatory factor for maturing thymocytes (MacNeil et al., 1990). IL-10 also enhances the proliferation of mast cells (Thompson-Snipes et al., 1991). It exerts immunosuppressive effects on cells of the monocyte/ macrophage lineage (deWaal Malefyt et al. 1991 Ding and Shevach, 1992), and also on subsets of activated helper

$CD4^+$ T cells (deWaal Malefyt et al., 1993, Taga and Tosato 1992), which are normally found in a quiescent or resting G_0 state before activation. The IL-10 receptor has been found on macrophages and B cells as well as T cells including murine $CD4^+$ T-cell clones (Taga et al., 1993, Lui et al., 1994 Weber Nordt et al , 1994) Significant levels of IL-10 receptor mRNA has also been detected in human $CD4^+$ T-cell clones (Liu et al , 1994) The IL-10 receptor is a member of the class II (interferon-like) subgroup of the cytokine receptor family and has been found to have a high affinity for the IL-10 molecule (Liu et al , 1994, Tan et al., 1993).

Table 1 Biological Properties of IL-10

Immunosuppressive

- Inhibits macrophage-dependent cytokine synthesis by T_H1 cells
  - (IFN-γ, IL-2, TNF-α, IL-3, and GM-CSF)
- Inhibits cytokine synthesis by activated macrophages
  - (IL-1α, GM-CSF, TNF-α, IL-6, IL-8, IL-10, and IL-12)
- Inhibits monocyte/macrophage MHC class II expression
- Inhibits monocyte/macrophage-dependent proliferation of T_H0 T_H1 and T_H2 cells
- Inhibits macrophage production of reactive nitrogen oxides
- Inhibits macrophage intracellular and extracellular killing of parasites

Immunostimulatory

- Enhances B cell MHC class II expression
- Enhances B cell viability
- Enhances activated B cell proliferation and antibody secretion
- Enhances cytotoxic T cell development
- Growth costimulator for thymocytes
- Growth costimulator for mast cells

Superantigens

In this study, I have used the superantigen staphylococcal enterotoxin B (SEB) as the mitogen to activate $CD4^+$ T cells. Superantigens are small, single chain proteins produced by a variety of bacteria, most notably *Staphylococcus aureus*, and by some viruses. They were recognized as having the ability to induce lymphocyte proliferation (Leavy et al., 1970). Their ability to stimulate T cell proliferation and cytokine production at concentrations as low as 10^{-16} mol/L (Langford et al., 1978) makes these proteins among the most powerful T cell activators known. They possess the unique ability to stimulate a large percentage of T cells, as much as 20% (Johnson et al., 1991) thereby causing the production of large amounts of inflammatory cytokines such as interleukin 2 (IL-2) and interferon-gamma (IFN-γ) (Carlsson and Sjorgen, 1985; Johnson and Magazine, 1988).

Superantigens, as potent T-cell-specific mitogens, activate the T-cell receptor in a major histocompatability complex (MHC) class II restricted manner (Johnson et al., 1991). Whereas "normal" antigens are processed by the antigen presenting cell (APC) and presented as a small peptide on the inside of the antigen-binding cleft of the MHC class II molecule, superantigens are not processed. Superantigens bind as intact proteins (Fleischer and Schrezenmeier, 1988) to the MHC class II molecule on the α-helical portions of the α and β chains outside of the antigen-binding cleft (Russell et al., 1991; Herman et al., 1991). It is in this manner that the superantigen is presented to the T cell (Figure 1). The superantigen also binds to the outside of the antigen-binding groove on the T cell receptor (TCR) specifically to the variable region of the beta chain (Pontzer et al., 1992). By binding outside the antigen-binding clefts on both the MHC class II

molecule and the TCR, superantigens bypass the normal antigen specificity required for T cell activation. It is for these reasons that superantigens can stimulate such a large percentage of T cells and can cause such dramatic responses in the immune system.

It has been shown that superantigens can directly activate T cells in the absence of APC's and MHC class II molecules (Taub and Rogers, 1992) (Figure 2). Binding of superantigens directly to the TCR without the presence of MHC class II molecules requires greater concentrations of superantigen. Without the stabilizing effect of the MHC class II molecule, thus binding of the superantigen to the TCR is less energetically favorable. Nevertheless, the same effects on T cells are observed: proliferation and cytokine production. SEB more closely resembles conventional antigens in terms of T-cell activation when compared to mitogens such as Concanavalin A (ConA) or phytohemagglutinin (PHA) since the activation signal is effected through the T cell receptor.

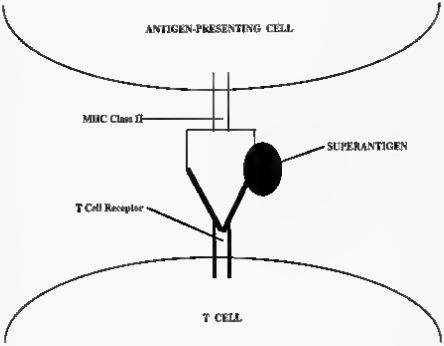

Figure 1· Superantigen binds to the MHC class II molecule outside of the antigen binding cleft as well as binding to the T cell receptor, also on the outside of the antigen recognition cleft.

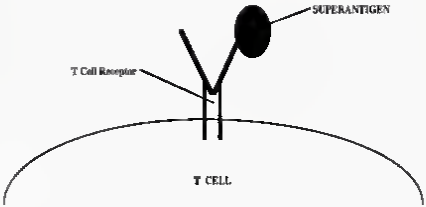

Figure 2: Binding of superantigen directly to the T cell receptor in the absence of MHC class II molecule

IL-10 in Health and Disease

Since IL-10 exhibits a wide range of biological activities, it has many potential uses in the clinical setting. The ability of IL-10 to suppress the production of inflammatory cytokines in monocytes suggests that it may be a potent anti-inflammatory reagent useful in the treatment of bacterial sepsis, rheumatoid arthritis (Isomaki et al., 1996) and psoriasis. IL-10's ability to suppress T_H1 derived inflammatory cytokines make it an attractive candidate for prolonging allograft survival, and in treating a variety of T-cell mediated autoimmune disorders, such as multiple sclerosis and type 1 diabetes. Interferon β has recently shown promise as a treatment for multiple sclerosis, and this interferon has been shown to induce the production of IL-10 in multiple sclerosis patients, possibly contributing to interferon β's effectiveness (Rudick et al., 1996). IL-10 has also been shown to restore tolerance to intestinal flora in a mouse model of inflammatory bowel disease (Duchmann et al., 1996), indicating the use of IL-10 as a possible treatment for ulcerative colitis and Crohn's disease in humans.

There are also cases where the biological activities of IL-10 may prove undesirable and where an antagonist of IL-10's activity may have potential clinical applications. IL-10 antagonists may provide effective therapy in infectious disease situations where IL-10 has been shown to inhibit macrophage-mediated immunity against intra- and extracellular parasites.

In order to study the safety of giving IL-10 as a therapeutic agent, recombinant human IL-10 was administered intravenously in a single dose to normal, healthy human volunteers at various concentrations. IL-10 was well tolerated, with only mild-to-moderate flu-like symptoms reported at the highest doses given (Huhn et al., 1996).

Furthermore, there were no clinically significant changes in the clinical chemistry, urinalysis or serology of the patients

Mammalian Cell Cycle

The mammalian cell cycle consists of five phases. G_0, G_1, S, G_2, and M (reviewed in Grana and Reddy, 1995) The G_0 phase represents the quiescent phase where cells are considered to "exit" the cycle and remain in a non-replicating, resting state Once the cell is stimulated by growth factors or mitogens to re-enter the cell cycle, it progresses into the G_1 phase where it prepares for DNA replication In the S phase, cellular DNA is replicated. The G_2 phase prepares the cell for mitosis, which is followed by the M phase where the cell physically divides. A multitude of cell proteins and biochemical pathways coordinate the cell cycle and the regulation of the system is complex

The progression of the cell cycle is directly dependent upon the activity of a set of protein kinases known as the cyclin-dependent kinases (cdks) As the name suggests, cdks are kinases which require regulatory subunits called cyclins in order to be active (Morgan, 1995). Cyclins are named for their cyclic nature of expression (Figure 3). They are specifically expressed during each phase of the cell cycle and are promptly degraded as the cycle advances (reviewed in Koff et al., 1992 Sherr 1994) These cdk-cyclin partners phosphorylate proteins which cause a cascade of events resulting in the coordination of cell cycle progression. Cell cycle control is carried out

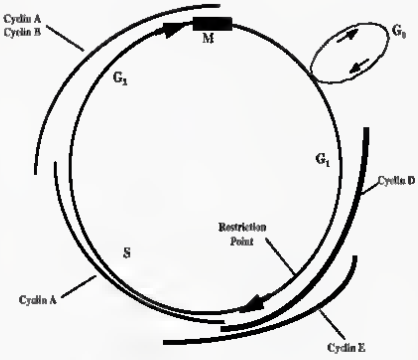

Figure 3: The Mammalian Cell Cycle.

in large part by regulating cdk activity using a variety of mechanisms and feedback loops

One group of proteins that is directly involved in the regulation of cdk activity are the cyclin-dependent kinase inhibitors (CKIs) (Elledge and Harper, 1994). These inhibitors are induced in response to specific extracellular signals and play an important role, via the inhibition of cdk activity, in blocking the cell cycle (Elledge and Harper, 1994; Pines, 1994). CKIs inhibit cdk function by physically binding cdk-cyclin complexes and interfering with kinase activity. Currently CKIs are separated into two families. The first includes $p21^{WAF1}$ and $p27^{KIP1}$ which share partial identity and are involved primarily with blocking G_1 and S phases of the cell cycle (Harper et al., 1993; Toyoshima and Hunter 1994; Xiong et al., 1993). Both $p21^{WAF1}$ and $p27^{KIP1}$ are present at high levels in quiescent cells and can cause G_1 arrest. $p21^{WAF1}$ was the first CKI to be identified and is considered to be a universal CKI (El-Deiry et al., 1993; Gu et al., 1993). $p27^{KIP1}$ has been found to be induced in response to TGF-β (Polyak et al., 1994).

In the entry of quiescent T cells from the G_0 phase into the G_1 phase of the cell cycle, two proteins act as mitogenic sensors. These are the CKI $p27^{Kip1}$ and the D-type cyclins (Sherr et al., 1994). These proteins act in a reciprocal fashion. Mitogenic stimulation causes the destruction of the inhibitor $p27^{Kip1}$ and the upregulation of cyclins D2 and D3. Cyclin D2 expression occurs early in the G_1 phase and is followed later in G_1 by the expression of cyclin D3 (Roberts et al. 1994). The inhibitor $p27^{Kip1}$ itself inhibits the kinase activity of complexes formed between the D cyclins and select cdks. These cyclin-cdk complexes are responsible for the phosphorylation of key substrates required for continued G_1 progression, an important one being the tumor

suppressor retinoblastoma protein pRB (Sherr et al., 1994). In general, the expression profiles of $p27^{Kip1}$ and the D-type cyclins are faithful markers of the activation state of T cells. In $CD4^+$ T cells, the downregulation of $p27^{Kip1}$ and the upregulation of D-type cyclins upon mitogenic stimulation are dependent on the binding of IL-2 to its receptor (Nourse et al., 1994). The importance of $p27^{Kip1}$ is underscored by the fact that immunosuppressive drugs like rapamycin that prevent the activation of T cells do so by preventing the downregulation of $p27^{Kip1}$ that occurs upon mitogenic stimulation (Nourse et al., 1994).

T-Cell Receptor Signaling

In order for a T cell to become activated from a quiescent state to a proliferative state, an activation signal must pass from the exterior of the cell through the T cell receptor to the proper components on the interior of the cell. The T cell receptor is a complex group of proteins consisting of multiple membrane-spanning proteins which interact with intracellular protein kinases and a series of adaptor proteins that relay kinase-derived signals to appropriate downstream effector pathways (Weiss, 1993, Alberola Ila et al., 1997). This sequence of events is set in motion when an antigen binds to the T cell receptor.

In the presence of antigens being presented in the context of MHC class II molecules, antigen-specific T cell receptors aggregate with each other on the surface of the T cell. Optimal signaling also requires the presence of T cell co-receptors such as CD4 molecules and the CD45 phosphatase as well as signaling through costimulatory

molecules such as CD28. The intracellular components of the T cell receptor complex and the co-receptors are brought into close proximity to one another upon T cell receptor binding. This allows the protein tyrosine kinase lck, which is associated with the CD4 co-receptor, and the non-receptor-associated tyrosine kinase fyn to then become activated by the CD45 phosphatase. It is believed that the CD45 phosphatase removes inhibitory phosphate groups from lck and fyn, thereby activating them (reviewed in Okumura and Thomas, 1995). Once activated, lck and fyn phosphorylate residues on the cytoplasmic tails of CD3 molecules, in particular the CD3 zeta chain. When the CD3 zeta chain becomes phosphorylated, it allows the 70-kilodalton zeta-associated protein (ZAP-70) to become activated. From this point, ZAP 70 helps regulate a number of signaling cassettes which each have different effects and components (reviewed in Berridge 1997). The inositol 1,4,5-triphosphate/ calcium cassette is initiated by phospholipase C and is responsible for regulating the levels of calcium necessary for a sustained signal from the T cell receptor to the cell nucleus. The diacylglycerol/ protein kinase C cassette has a major role in stimulating gene transcription which is essential for the T cell to become activated. The precise function of the phosphatidylinositol 3-kinase signaling cassette is not well known but seems to be related to a number of downstream processes in T cell activation. One of the main signaling cassettes is the ras/ mitogen-activated protein (MAP) kinase pathway (recently reviewed in Schaeffer and Weber, 1999).

MAP Kinase Pathway

The MAP kinase pathway is initiated by the activation of $p21^{Ras}$ by receptor tyrosine kinases and coordinated by adaptor proteins. Ras is a GDP/GTP regulated protein that resides at the inner surface of the plasma membrane. The receptor tyrosine kinases activate a group of adaptor proteins, including Grb and human SOS, that act as guanine nucleotide exchange factors. Activation of these proteins promotes the formation of the active, GTP bound form of Ras (reviewed in Vojtek and Der, 1998). The active form of Ras then brings the first member of the serine/ threonine kinase pathway, Raf, into close proximity to the plasma membrane where it is phosphorylated and thereby activated. Once activated, Raf then goes on to activate MEK-1 and MEK-2, which both in turn phosphorylate the extracellular signal-regulated kinases (ERK) ERK-1 and ERK-2. Activated ERKs translocate to the nucleus and directly phosphorylate transcriptional regulatory proteins (Figure 4), including c-fos, c-jun and transcription factors such as Elk-1 (Schaeffer and Weber, 1999). Thus, the activation signal from the T cell receptor enters the nucleus, inducing the production of IL-2 and activating transcription factors that will allow for the proliferation of the T cell.

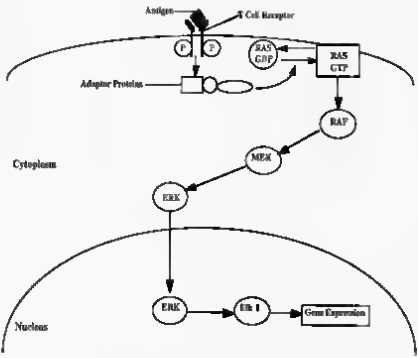

Figure 4: MAP Kinase pathway

CHAPTER 2
MATERIALS AND METHODS

Isolation of Peripheral Blood Mononuclear Cells and Cell Culture

Human cells were collected from the whole blood of normal healthy volunteers or from leukocyte source packs (Civitan Regional Blood Center, Gainesville, FL). Peripheral blood mononuclear cells (PBMC) were isolated by Histopaque (Sigma, St. Louis MO) density gradient centrifugation (Torres and Johnson, 1994) and viability was determined to be greater than 95% by trypan blue exclusion. For isolation of $CD4^+$ T cells, PBMCs were loaded onto CD4 T Cellect columns (Cytovax, Edmonton, Alberta, Canada) as per the manufacturers recommendation and cell purity was assessed by flow cytometry. Cells were maintained in RPMI 1640 supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS) unless otherwise noted, 200 units/ml penicillin and 200 mg streptomycin in a 5% CO2 atmosphere at 37° C and used immediately.

Mixed Lymphocyte Reaction and Proliferation Assay

The effect of IL-10 on the proliferative response of responder PBMCs to cells from an allogeneic donor was determined by culturing the PBMCs from the responder

with mitomycin C-inactivated PBMCs from another donor and measuring the incorporation of [^{3}H]thymidine into DNA. Specifically, 4 x 10^4 PBMCs from one donor were added per well in a round bottom 96-well plate either in the presence or absence of IL-10 at 10 units/ml (Intergen, Purchase, NY). These responder cells were incubated at 37^{0}C with or without IL-10 for 2 hours prior to the addition of the stimulator cells. Stimulator PBMCs were prepared from another donor by incubating these cells in a 60 µg/ml mitomycin C solution for 30 minutes at 37^0 C. These cells were then washed with culture media resuspended in culture media and incubated for another 30 minutes. These cells were then washed again in culture media and 4 x 10^4 of these cells were co-cultured with the responder cells for 5 days at 37^{0}C. Next, 1 µCi of [^{3}H]thymidine (Amersham, Arlington Heights, IL) in media was added per well and the plates incubated for 8 hours before harvest. [^{3}H]thymidine incorporation was measured as counts per minute (CPM) on a liquid scintillation counter (Becton Dickinson, San Jose, CA). All experiments were performed in quadruplicate.

The proliferative response of PBMCs to staphylococcal enterotoxin B (SEB) (Toxin Technology, Sarasota, FL) was also determined by the incorporation of [^{3}H]thymidine into DNA. Specifically, PBMCs were added to wells in a 96-well plate at a concentration of 3 x 10^5 cells/ml and were treated with either IL-10 at 10 units/ml or media alone and incubated for 48 hours. Media or SEB at concentrations from 10 pg/ml to 100 ng/ml was then added at the indicated concentrations and the cultures were incubated for another 48 hours in a final volume of 200 µL/well. Next, 1 µCi of [^{3}H]thymidine (Amersham) in media was added per well and the plates incubated for 8 hours before harvest. [^{3}H]thymidine incorporation was measured as counts per minute

(CPM) on a liquid scintillation counter (Becton Dickinson). All experiments were performed in quadruplicate. The proliferative response of purified $CD4^+$ T cells to SEB was performed in a similar manner, except that cells were added to the plates at a concentration of 2.5 x 10^6 cells/ml. This was necessary to obtain substantial proliferation, as determined by experimentation. These cells were only incubated with IL-10 for 2 hours before the addition of SEB.

Flow Cytometry

PBMCs were added to 6-well plates at a concentration of 3 x 10^6 cells/ml and treated with IL-10, SEB and/or IL-2 (Intergen) at the indicated concentrations in a final volume of 5 ml. Plates were incubated for the time indicated before harvest for flow cytometry analysis. At harvest, cells were gently mixed and cell counts were performed. Cells (1 x 10^6) were added to a 15-ml tube and washed twice with 12 ml cold standard azide buffer [phosphate-buffered saline (PBS) containing 5% (v/v) FBS and 0.1% (w/v) sodium azide], centrifuging each time at 500 RPM for 10 min at 4° C. After carefully removing the supernatant, the cells were adjusted to 1 x 10^7 cells/ml by resuspending the pellet in 80 µl cold standard azide buffer and adding 20 µl fluoresceon isothiocyanate (FITC)-labeled anti-CD4 antibody (Pharmingen, San Diego, CA) and incubating on ice for 30 min in the dark. Cells were then washed twice as described before. For propidium iodide staining, the cells were then resuspended in 1 ml of a 0.112% sodium-citrate buffer containing 50 µg/ml propidium iodide (Sigma) and 100 units/ml RNase A (Sigma) and allowed to stain for up to 1 hour at room temperature. Propidium iodide

stains cellular DNA and allows the quantitation of the relative amount of DNA present in each cell. This information is then used to calculate the number of copies of DNA in the cell and to pinpoint which phase of the cell cycle the cell is currently occupying.

For intracellular staining of c-fos and c-jun proteins, the cells were harvested as before, washed with cold staining buffer [PBS containing 1% (v/v) FBS and 0.1% (w/v) sodium azide] and stained with FITC-labeled anti-CD4 antibody in staining buffer as described before. After washing twice with cold staining buffer and once with cold PBS, the cells were fixed with 100 µl of 4% (w/v) paraformaldehyde in PBS for 20 min at 4°C. The cells were then washed once with cold PBS and twice with cold permeabilization buffer [staining buffer with 0.1% (w/v) saponin]. The cells were then resuspended in 100 µl of permeabilization buffer containing 0.7 µg of either c-fos or c-jun specific rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) on ice for 30 min. The cells were then washed twice with permeabilization buffer and resuspended in permeabilization buffer containing 0.7 µg of phycoerythrin-labeled anti-rabbit antibody (Sigma) on ice for 30 min. The cells were again washed twice with permeabilization buffer and washed once with staining buffer. Finally, the cells were resuspended in 1ml of staining buffer.

For all flow cytometry analysis, samples were filtered through 44 µm nylon mesh and data from 30,000 events were acquired with a FACSort (Becton Dickinson Immunocytometry Systems, San Jose, CA) using the LYSIS II software system. Analysis of the cell cycle was performed using CellFIT software (Becton Dickinson, San Jose, CA).

Immunoblotting

PBMCs were cultured in 6-well plates and harvested as described above $CD4^+$ T cells were isolated by passing PBMCs over CD4 T Cellect columns (Cytovax, Edmonton, Alberta, Canada) as per the manufacturer s recommendations The resulting $CD4^+$ T cells were counted and stored at -70^0 C

Cells were lysed at 4^0 C for 20 min in ice-cold lysis buffer consisting of 50 mmol/L Tris-HCl (pH 7 5), 250 mmol/L NaCl, 1% (v-v) Triton X 100 2 mmol/L EDTA, 2 mmol/L EGTA, 50 mmol/L NaF, 20 mmol/L β-glycerophosphate, 2 mmol/L Na_3VO_4, aprotinin (10 μg/ml), leupeptin (10 μg/ml), pepstatin (10 μg/ml), benzamidine (5 μg/ml) and 1 mmol/L phenylmethanesulfonyl fluoride (PMSF) Samples were centrifuged at 13,000 RPM for 10 min and a BCA protein assay (Pierce, Rockford, IL) was performed on the supernatants. Equal amounts of protein from cell lysates (30-70 μg/ lane) were subjected to sodium dodecyl sulfate (SDS) gel electrophoresis. After Western transfer, membranes were probed with 1.2 μg of anti-$p27^{KIP1}$, anti-cyclin D2, or anti-cyclin D3 antibodies (Santa Cruz Biotechnology) and developed using an enhanced chemiluminescence (ECL) detection kit (Amersham) Densitometric analysis of radiographic film using iA-1000 Digital Analysis Software (Alpha Innotech Corp, San Leandro, CA) was used to determine fold increase or decrease between band intensities based on total pixel value.

Interleukin-2 Assay

IL-2 levels were determined from cell-free culture supernatants stored at –70° C using IL-2 enzyme-linked immunosorbent assay (ELISA) kits (Intergen) Assay protocol was conducted as described by the manufacturer All supernatant samples were tested in duplicate

Extracellular Signal-Regulated Kinase Assay

$CD4^+$ T cells were isolated as described previously and incubated in media without FBS for 1-2 hours to decrease basal levels of activity prior to use 1×10^7 cells were added to 1 5 ml microfuge tubes and incubated with or without IL-10 at 100 units/ml for 20 minutes at 37°C These cells were then stimulated as described for the times indicated They were then centrifuged at 2000 RPM for 10 seconds, the supernatant was removed and the cells were quick-frozen in liquid nitrogen before storage at -70°C until use Cells were then lysed at 4° C for 20 min in ice-cold NP-40 lysis buffer consisting of 50 mmol/L Tris-HCl (pH 7 5), 250 mmol/L NaCl, 1% (v v) Triton X-100, 2 mmol/L EDTA, 2 mmol/L EGTA, 50 mmol/L NaF, 20 mmol/L β-glycerophosphate, 2 mmol/L Na_3VO_4, aprotinin (10 μg/ml) leupeptin (10 μg/ml) pepstatin (10 μg/ml), benzamidine (5 μg/ml) and 1 mmol/L PMSF. Samples were centrifuged at 13,000 RPM for 10 min, a BCA protein assay (Pierce, Rockford, IL) was performed on the supernatants and the samples were normalized for protein content Antibodies to ERK-1 and ERK-2 (1 μg each, Santa Cruz Biotechnology) were added to the supernatants and the samples were immunoprecipitated overnight at 4°C Next, 40

µL of a 1:1 Protein A-Sepharose (Sigma) slurry was added to each sample and they were incubated for 2 hours on a nutator at 4°C. The samples were then centrifuged at 2000 RPM for 10 seconds and the supernatants removed. The immunoprecipitates were then washed twice with cold lysis buffer and twice with cold kinase buffer (20 mmol/L MOPS (pH 7.2), 2 mmol/L EGTA, 20 mmol/L $MgCl_2$, 1 mmol/L DTT) before being resuspended in 30 µL kinase buffer. Then the following was added to each sample: 0.5 µg of ATP, 10 mCi of [γ-^{32}P]-ATP (Amersham) and 6 µg of myelin basic protein (MBP) and the samples were incubated for 20 minutes at 35°C. The samples were eluted in SDS-PAGE sample buffer, separated by SDS-PAGE and visualized by autoradiography.

Raf-1 Kinase Cascade Assay

$CD4^+$ T cells (1×10^7) were isolated as described previously, serum-starved, incubated with or without IL-10 (100 units/ml), stimulated and frozen as described above. These cells were then lysed for 20 minutes at 4°C in 400 µL of lysis buffer containing 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 0.1 % SDS, 1 % (v:v) Triton X-100, 2 mmol/L EDTA, 5 mmol/L NaF, 0.1 % (v:v) β-mercaptoethanol, 1 mmol/L Na_3VO_4, 1 mmol/L PMSF and 2 µg/ml each of aprotinin and leupeptin giving a protein concentration of roughly 1 mg/ml for 1×10^7 cells. The lysates were then centrifuged at 13 000 RPM for 10 minutes and separated from the cellular debris. Antibodies to Raf-1 (3 µg, Santa Cruz Biotechnology) were added to the supernatants and the samples were immunoprecipitated for 1 hour at 4°C. Next, 75 µL of a 1:1

Protein A-Sepharose slurry was added to each sample and they were incubated for 2 hours on a nutator at 4°C. The samples were then centrifuged at 2000 RPM for 10 seconds and the supernatants removed. The immunoprecipitates were then washed twice with cold lysis buffer and twice with cold kinase buffer (20 mmol/L MOPS [pH 7.2], 25 mmol/L β-glycerophosphate, 5 mmol/L EGTA, 1 mmol/L Na_3VO_4 and 1 mmol/L DTT) before being resuspended in 15 µL kinase buffer. Then the following was added to each sample: 1 µg of unactive MEK-1 (Upstate Biotechnology, Upsala NY), 2.5 µg of unactive ERK-2 (Upstate Biotechnology) and 10 mL of Mg/ATP cocktail (75 mmol/L $MgCl_2$ and 500 µmol/L ATP in kinase buffer). The samples are then incubated for 30 minutes at 30°C and then centrifuged at 14,000 RPM for 15 seconds. Then, 10 µl of this supernatant is incubated in another microfuge tube with 5 µg of Elk-1 (Upstate Biotechnology), 10 µCi of [γ^{32}P]-ATP (Amersham) in Mg/ATP cocktail and the volume is adjusted to a total of 30 µL with kinase buffer. The samples were incubated for 30 minutes at 30°C, eluted in SDS-PAGE sample buffer, separated by SDS-PAGE and visualized by autoradiography.

CHAPTER 3

RESULTS

Suppression of the Mixed Lymphocyte Reaction

IL-10 was tested for its ability to suppress the Mixed Lymphocyte Reaction (MLR). Responder PBMCs were pretreated with IL-10 and then cultured for 5 days in the presence of mitomycin C-inactivated PBMCs from another donor. The subsequent proliferative response was determined by measuring [^{3}H]thymidine incorporation. IL-10 was shown to suppress responder cell proliferation to varying degrees. With some responder cells, the IL-10 induced suppression of the MLR was over 30%, while with responder cells from other donors this IL-10 induced suppression was negligible at just over 5% (Figure 5). This variability was due to a number of factors inherent in the MLR system. Most notably was the variation in the strength of reaction between responder and stimulator cells which was itself dependent upon differences between responder and stimulator cell MHC molecules. While some donor cells responded strongly to the stimulator cells, others failed to mount a productive response (data not shown). Due to these widely variable responses, it was determined that another system be found to evaluate IL-10's immunosuppressive effects.

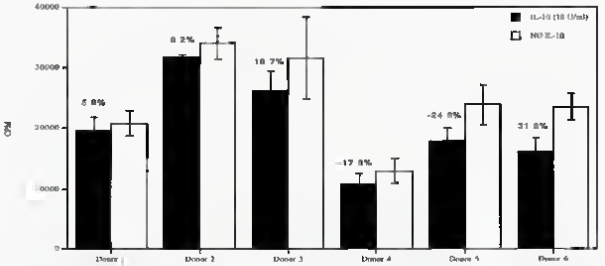

FIGURE 5. IL-10 Suppression of the Mixed Lymphocyte Reaction. Responder PBMCs from one donor were added to round bottom 96-well plates 4×10^4 cells/well in the presence or absence of IL-10 at 10 units/ml and incubated for 2 hours. Next, the same number of stimulator PBMCs from another donor were added to the wells and cultured for 5 days at 37°C. Proliferation was measured by [^{3}H]thymidine incorporation. This figure summarizes the proliferative response of different responder cell donors. Data are expressed as the mean CPM±SD. Numbers above graphs represent percent suppression by IL-10.

Inhibition of SEB-Induced Proliferation in PBMCs

IL-10 was next tested for its ability to inhibit superantigen-induced proliferation of human lymphocytes. Human PBMCs were treated with IL-10 for up to 2 days before the addition of SEB. These cultures were then incubated for up to 5 days, which was the time of optimal proliferation as reflected by [^{3}H]thymidine incorporation. IL-10 significantly inhibited SEB-induced proliferation in a dose-dependent manner (data not shown), and an optimal concentration of 10 units/ml of IL-10 was determined. Furthermore, as shown in Figure 6, 10 units/ml of IL-10 inhibited PBMC proliferation over a wide range of concentrations of SEB. Thus, IL-10 blocked SEB-induced PBMC proliferation.

Inhibition of Cell Cycle Progression in SEB-Stimulated CD4$^+$ T Cells

I next determined the stage of the cell cycle in which IL-10 blocked SEB-induced proliferation, looking specifically at CD4$^+$ T cells, which are one of the primary targets of superantigen stimulation. After stimulation for up to 5 days, CD4$^+$ T cells were examined for cell cycle progression using FITC-labeled anti-CD4 antibodies and propidium iodide staining. Preliminary experiments using SEB showed that CD4$^+$ T cells were entering the S phase between days 4 and 5, although there was some variation among donors with respect to the percentage of cells in S phase at these times. As shown in Table 2, untreated cells were all essentially quiescent (>98% G_0/G_1). Treatment with SEB induced cells to progress through the G_1 phase and into the S phase of the cell cycle. However, IL-10 blocked SEB-induced progression of CD4$^+$ T cells

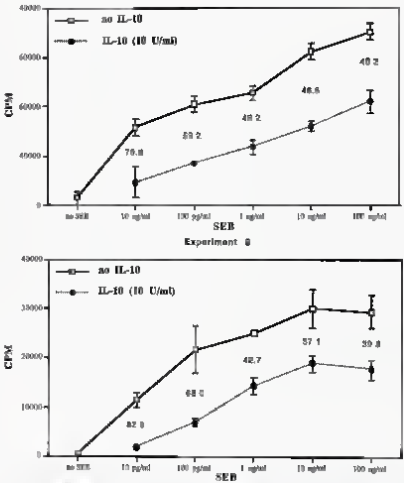

FIGURE 6: IL-10 suppression of SEB stimulation. SEB dose response PBMCs were added to 96-well plates at 3 x 10^5 cells/ml along with IL-10 at 10 units/ml or media only After 2 days of incubation, media alone or SEB at varying concentrations was added and plates were further incubated for another 2 days Proliferation was measured by [^{3}H]thymidine incorporation. Different PBMC donors are represented in each experiment Data are expressed as the mean CPM ± SD Results were determined to be statistically significant by the Student s t-test ($P \leq .002$) Numbers between graphs represent the percentage of suppression by IL-10

Table 2: Flow cytometry analysis of the effect of IL-10 on SEB stimulated CD4 T cells

Treatment	Cell cycle distribution (%) [a,b]		
	G_1	S	G_2/M
Day 4			
None	>98%	<1%	<1%
SEB only	58.80% ± 0.7	37.90% ± 0.3	3.25% ± 0.4
IL-10 + SEB	78.95% ± 2.2	19.00% ± 1.6	2.05% ± 0.6
Day 5			
None	>98%	<1%	<1%
SEB only	44.10% ± 1.0	47.15% ± 0.9	8.70% ± 0.1
IL-10 + SEB	72.95% ± 2.2	22.45% ± 1.8	4.70% ± 0.4

[a] PBMC were pretreated for two days with IL-10 (10 U/ml). SEB (100 pg/ml) was then added to these cultures and the incubation continued for the indicated number of days before flow cytometry analysis. Data was gated to analyze CD4 T cells. Results are expressed as the mean of duplicate samples ± s.d

[b] All differences between SEB only and IL-10+SEB treatments were determined to be statistically significant by Student's t-test ($p<0.01$).

through the G_1 phase of the cell cycle. A similar pattern of inhibition was observed with 3 and 30 units/ml of IL-10, with a resulting dose response (Table 3). These data suggest that IL-10 inhibits the early SEB-mediated activation of $CD4^+$ T cells and prevents their progression through the G_1 phase of the cell cycle and their subsequent entry into the cell cycle.

Time Course of Inhibition of Cell Cycle Progression

I then examined the inhibitory effect of IL-10 on SEB stimulation of PBMCs when added to cultures at various times relative to superantigen addition. As seen in Table 4, addition of IL-10 (10 units/ml) 2 days before, 1 day before, or at the time of SEB addition inhibited cell proliferation in the G_0/G_1 phase of the cell cycle to essentially the same extent. The addition of IL-10 at 1 to 4 days after SEB resulted in progressively less inhibition of cell cycling. The addition of IL-10 4 days after SEB allowed essentially the same percentage of cells to leave the G_1 phase and enter the cell cycle as did cells treated with SEB and no IL-10. These data suggest that, once $CD4^+$ T cells have been activated by mitogen and enter the cell cycle, IL-10 has no further effect on these cells. Thus, it appears that IL-10 blocks the activation of quiescent $CD4^+$ T cells but not the subsequent proliferation of these cells.

Table 3. Flow cytometry analysis of the effect of increasing concentrations of IL-10 on SEB stimulated CD4+ T cells

IL-10 (U/ml)	SEB	Cell cycle distribution (%) [a]		
		G_1	S	G_2/M
0	-	>98%	<1%	<1%
30	+	80.50%	16.05%	3.45%
10	+	75.10%	21.00%	3.85%
3	+	73.80%	21.60%	4.50%
0	+	61.50%	32.00%	6.50%

[a] PBMC were pretreated for two days with IL-10 at the indicated concentrations and then incubated with SEB (100 pg/ml) for 5.5 days before flow cytometry analysis. Data was gated to analyze CD4+ T cells.

Table 4: Flow cytometry analysis of the effect of IL-10 addition at various times relative to SEB incubation on SEB stimulated CD4+ T cells

Treatment	Cell cycle distribution (%) [a]		
	G_1	S	G_2/M
None	>98%	<1%	<1%
IL-10 added 2 days prior to SEB	84.50%	11.70%	3.80%
IL-10 added 1 day prior to SEB	88.30%	8.90%	2.80%
IL-10 added same time as SEB	83.50%	13.00%	3.50%
IL-10 added 1 day after SEB	72.10%	21.80%	6.00%
IL-10 added 2 days after SEB	70.90%	22.80%	6.30%
IL-10 added 3 days after SEB	70.80%	22.90%	6.30%
IL-10 added 4 days after SEB	65.80%	26.80%	7.40%
SEB only	66.15%	27.10%	6.75%

[a] PBMC were treated with IL-10 (10 units/ml) at times indicated and incubated with SEB (100 pg/ml) 5.5 days after the addition of SEB cells were collected and analyzed by flow cytometry Data was gated to analyze CD4+ T cells.

Maintenance of Tumor Suppressor Protein $p27^{Kip1}$ Levels

To gain insight into how IL-10 blocked superantigen-induced activation in the G_0/G_1 phase of the cell cycle, I determined the effects of IL-10 on the tumor suppressor gene protein $p27^{Kip1}$ and on the cyclins D2 and D3 in $CD4^+$ T cells. $p27^{Kip1}$ levels are high in quiescent G_0 cells and are rapidly reduced in cells induced to enter the cell cycle by mitogens (Pelyak et al., 1994), whereas cyclin D levels are low in quiescent cells and increase upon mitogenic stimulation (Ajchenbaum et al. 1993). In human T cells, cyclin D2 migrates as a doublet, with the faster migrating species representing the activated form (Modiano et al., 1994). PBMCs were incubated with IL-10 (10 units/ml) for 2 days before the addition of SEB. After incubating for the specified number of days, the $CD4^+$ T cells were isolated using negative selection chromatography columns and the cell extracts were subjected to Western analysis for $p27^{Kip1}$ and cyclins D2 and D3. As shown in Figure 7, untreated quiescent cells showed high levels of $p27^{Kip1}$. Treatment with SEB dramatically reduced the levels of $p27^{Kip1}$ by day 3.5. By comparison, the $p27^{Kip1}$ level remained relatively high in cultures treated with IL-10 even in the presence of SEB. IL-10 had no effect on another cell cycle inhibitory protein $p21^{Waf1}$ (data not shown). The effect of IL-10 on the expression of $p27^{Kip1}$ in SEB-treated cultures supports the conclusion that IL-10 inhibits cellular proliferation in the early G_0/G_1 phase of the cell cycle.

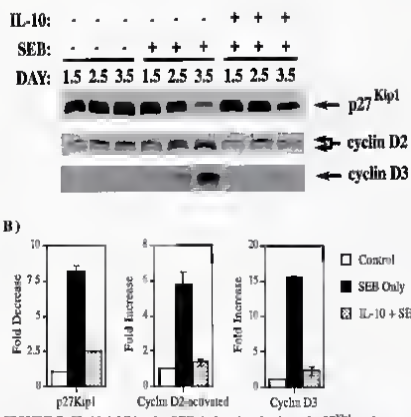

FIGURE 7 IL-10 inhibits the SEB induced reduction of $p27^{Kip1}$ and prevents SEB-induced upregulation of cyclins D2 and D3 in $CD4^+$ T cells. A) PBMCs were incubated in 6-well plates at 6×10^5 cells/ml along with IL 10 at 10 units/ml or media only After 2 days of incubation, media alone or SEB at 100 pg/ml was added. The cells were further incubated until the indicated harvest day Cells were harvested and $CD4^+$ T cells were separated and collected by negative selection affinity chromatography Lysates from these cells were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) and Western analysis using antibodies specific for $p27^{Kip}$, cyclin D2 and cyclin D3 The lower band in the cyclin D2 doublet represents the faster-migrating activated form of cyclin D2 B) Quantitation was performed by densitometric analysis of bands from day 3 5 The differences between SEB treatment and treatment with IL-10 and SEB were found to be statistically significant for all immunoblots ($P< 001$) by Student's t-test.

Inhibition of Cyclin Upregulation

The expression of cyclins D2 and D3 were also strongly affected by IL-10 treatment of SEB-stimulated $CD4^+$ T cells. Figure 7 shows that SEB treatment of quiescent $CD4^+$ T cells increased the levels of cyclin D3 and the activated faster migrating form of cyclin D2 by Day 3.5. In contrast, IL-10 prevented the expression ofcomparable amounts of both cyclin D3 and the faster migrating form of cyclin D2 over the same time period. As mentioned earlier $p27^{Kip1}$ and the D-type cyclins behave as sensors for mitogenic stimuli and play a crucial role in the decision of cells to become activated, enter the cell cycle, and subsequently proliferate. Taken together with the effects on $p27^{Kip1}$, these data demonstrate that IL-10 affects $CD4^+$ T cells very early in the cell cycle by antagonizing the ability of mitogens like SEB to activate these cells to enter the cell cycle.

IL-10 Inhibits IL-2 Production

The potent mitogenic activity of SEB requires the induction and autocrine effects of IL-2 for the activation of $CD4^+$ T cells. IL-2 is the primary cytokine secreted by $CD4^+$ T cells that is responsible for the activation of competent T cells and their subsequent progression through the G_1 phase of the cell cycle. IL-10 has also been reported to block induction of IL-2 (Fiorentino et al., 1989). Thus, I next examined the role of IL-2 in this system and its effects on the cell cycle events affected by IL-10. As seen in Table 5, treatment with IL-10 dramatically inhibited the amount of IL-2 produced during the time period of SEB stimulation. These data represent IL-2 levels in

Table 5: IL-10 effects on IL-2 production by SEB stimulated PBMC

Days	IL-10	SEB	IL 2 [a,b]		
			pg/ml	units/ml [c]	Fold decrease
1.5	-	-	12 ± 5.0	0.12 ± 0.050	-
2.5	-	-	<8 [d]	<0.08 [d]	-
3.5	-	-	<8	<0.08	-
1.5	-	+	1872 ± 185.8	18.72 ± 1.850	
2.5	-	+	2118 ± 246.0	21.18 ± 2.460	-
3.5	-	+	2277 ± 318.0	22.77 ± 3.180	
1.5	+	+	129 ± 6.0	1.29 ± 0.060	14.5
2.5	+	+	240 ± 29.0	2.40 ± 0.290	8.8
3.5	+	+	49 ± 7.0	0.49 ± 0.070	46.2

[a] IL-2 was measured from supernatants from PBMC cultures treated with media only, SEB (100 pg/ml) only or IL-10 (10 U/ml) for two days prior to SEB (100 pg/ml). Cell supernatants were then collected after the indicated incubation period

[b] Results were shown to be statistically significant by Student's t-test ($p<0.02$)

[c] IL-2 was measured in pg/ml. The specific activity of the IL-2 standard was 1×10^7 units/mg and was used to calculate units/ml.

[d] These results were below the detection limit of the assay.

supernatants taken from the same cell cultures analyzed in Figure 7. The changes in IL-2 levels are seen to precede changes in the cell cycle proteins. The cell cycle proteins analyzed are under the direct influence of IL-2. I then measured the amount of IL-2 produced during the first 24 hours after SEB stimulation (Table 6) to study the effect IL-10 has on IL-2 production during the time of initial T cell activation. IL-2 production was induced as early as 12 hours after the addition of SEB, and IL-10-treated cells showed significantly less IL-2 production than cells incubated with SEB only. In both cases, similar values were obtained when IL-10 was added 2 days before the addition of SEB and when IL-10 and SEB were added simultaneously (data not shown). Thus, IL-10 blocks induction of IL-2 in the SEB-stimulated cultures.

IL-2 Reverses IL-10 Effects on Cell Cycle Progression

Next, I determined the effect of the addition of IL-2 on the IL-10-induced suppression of SEB stimulation. IL-2 at varying concentrations was added to PBMCs along with IL-10 and SEB. The cells were then incubated for 5 days and the $CD4^+$ T cells were examined for cell cycle progression using FITC-labeled anti-CD4 antibodies and propidium iodide staining. Table 7 shows that the addition of IL-2 overcame the suppressive effect of IL-10 in these cells and allowed them to become activated and progress from the G_0/G_1 phase into S and G_2/M phases of the cell cycle. This reversal of the IL-10 effect by IL-2 occurred in a dose-dependent manner. A similar experiment was performed in which cells were incubated with IL-10 for 2 days before the addition of IL-2 and SEB (Table 8). Results very similar to those in Table 7 were seen in which

Table 6: Early IL-10 effects on IL-2 production by SEB stimulated PBMC

Hours	IL 10	SEB	IL-2 [a,b]		Fold decrease
			pg/ml	units/ml [c]	
6	-	-	<8 [d]	<0.08 [d]	-
12			<8	<0.08	
18			<8	<0.08	
24			<8	<0.08	
6		+	<8	<0.08	
12		+	172 ± 20.8	1.72 ± 0.208	
18		+	816 ± 17.0	8.16 ± 0.170	
24		+	1375 ± 18.6	13.75 ± 0.186	
6	+	+	<8	<0.08	0
12	+	+	53 ± 6.0	0.53 ± 0.060	3.2
18	+	+	53 ± 4.7	0.53 ± 0.047	15.4
24	+	+	96 ± 12.0	0.96 ± 0.129	14.3

[a]IL-2 was measured from supernatants from PBMC cultures treated with media only SEB (100 ng/ml) only or IL-10 (10 U/ml) for two days prior to SEB (100 ng/ml). Cell supernatants were then collected after the indicated incubation period.

[b]Results were determined to be statistically significant by Student's t-test ($p<0.05$)

[c]IL-2 was measured in pg/ml. The specific activity of the IL-2 standard was $1x10^7$ units/mg and was used to calculate units/ml.

[d]These results were below the detection limit of the assay

Table 7- Flow cytometry analysis of the effect IL-2 addition on reversing the effect of IL-10 on SEB stimulated CD4 T cells. NO IL-10 pretreatment.

IL-2 (U/ml)	IL 10	SEB	Cell cycle distribution (%) [a]		
			G_1	S	G_2/M
0	-	-	>98%	<1%	<1%
0	-	+	63 60%	29 10%	7 10%
100	+	+	59 70%	33 40%	6 90%
30	+	+	60.60%	31 90%	7.50%
10	+	+	72 30%	22 10%	5.60%
3	+	+	77.30%	17 60%	4.80%
0	+	+	80.50%	15.00%	4.50%
100	-	+	48.60%	42 70%	8.70%
3	-	+	59 50%	32.30%	8 10%
100	+	-	94 60%	5 00%	0 60%
3	+	-	98 40%	1 60%	0 20%
100	-	-	92 70%	6 30%	1 00%
3	-	-	97 60%	2.00%	0.40%

[a] PBMC were treated simultaneously with IL-10 (10 U/ml), SEB (100 pg/ml) and IL-2 at the indicated concentrations for 5.5 days before flow cytometry analysis. Data was gated to analyze $CD4^+$ T cells.

Table 8: Flow cytometry analysis of the effect IL-2 addition on reversing the effect of IL-10 on SEB stimulated CD4+ T cells. 2 day IL-10 pretreatment.

IL-2 (U/ml)	IL-10	SEB	Cell cycle distribution (%) [a]		
			G_1	S	G_2/M
0	-	-	>98%	<1%	<1%
0	-	+	73.70%	23.70%	3.10%
100	+	+	61.20%	33.70%	5.60%
30	+	+	62.10%	32.60%	5.30%
10	+	+	79.50%	16.00%	4.50%
3	+	+	88.30%	9.50%	2.20%
0	+	+	89.20%	8.70%	2.00%
100	-	+	53.80%	41.20%	5.80%
3	-	+	70.80%	24.70%	4.50%
100	+	-	80.80%	16.80%	2.40%
3	+	-	93.30%	5.50%	1.10%
100	-	-	65.70%	29.40%	4.90%
3	-	-	74.90%	23.80%	2.20%

[a] PHMC were treated for 2 days with IL-10 (10 U/ml). They were then incubated with SEB (100 pg/ml) add IL-2 at the indicated concentrations for 5.5 days before flow cytometry analysis.
Data was rated to analyze CD4+ T cells.

the addition of IL-2 reversed the G_0/G_1 block imposed by IL-10 in a dose-dependent manner. In either case, the concentration of IL-2 required to produce an approximately 50% reversal of inhibition was similar to the concentration of IL-2 induced in cultures stimulated by SEB alone (see Table 5). These IL-2 levels probably represent steady-state levels of IL-2 in culture supernatants. These data strongly support the conclusion that one important mechanism by which IL-10 exerts its effects is by downregulating the production of IL-2, especially since pretreatment with IL-10 had no greater effect on the ability of IL-2 to reverse the cell cycle block. In a few donors tested, as shown in Table 8, IL-10 also inhibited cell cycle progression in cells stimulated with exogenous IL-2, indicating that IL-10 may also interfere with the response of $CD4^+$ T cells to IL-2 to varying extents.

IL-2 Partial Reversal of IL-10 Effects on Cell Cycle Proteins

I next examined the effect of adding exogenous IL-2 on the levels of the cell cycle proteins $p27^{Kip1}$ and cyclin D2 in $CD4^+$ T cells. I concentrated on $p27^{Kip1}$ and cyclin D2, because changes in $p27^{Kip1}$ and cyclin D2 occur very early in G_1 as compared to cyclin D3. PBMCs were treated simultaneously with IL-10, SEB and IL-2. The $CD4^+$ T cells were then isolated and the cell extracts were subjected to Western analysis as described before. As seen in Figure 8, $p27^{Kip1}$ levels again decreased in SEB-treated cultures by day 3.5 and remained high in cultures treated with IL-10, as described earlier (see Figure 7). The addition of IL-2 along with the IL-10 and SEB caused the $p27^{Kip1}$ levels to decrease to nearly those seen in cells treated with SEB only. Figure 8 also

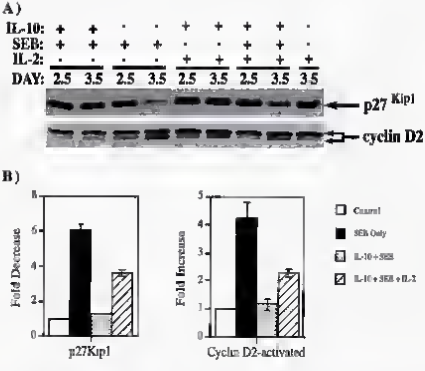
A)
IL-10: + + - - + + + + -
SEB: + + + + - - + + -
IL-2: - - - - + + + + +
DAY: 2.5 3.5 2.5 3.5 2.5 3.5 2.5 3.5 3.5
p27 Kip1
cyclin D2
B)
Fold Decrease
p27Kip1
Fold Increase
Cyclin D2-activated
Control
SEB Only
IL-10 + SEB
IL-10 + SEB + IL-2

FIGURE 8 Addition of IL-2 partially reverses the effects of IL-10 on the SEB-induced reduction of $p27^{Kip1}$ and SEB-induced upregulation of cyclin D2 in $CD4^+$ T cells. (A) PBMCs were incubated in 6 well plates at 6×10^5 cells/ml along with IL-10 at 10 units/ml or media only. After 2 days of incubation, media alone, SEB at 100 pg/ml, or SEB and IL-2 (100 units/ml) were added. The cells were further incubated until the indicated harvest day. Cells were harvested and $CD4^+$ T cells were separated and collected by negative selection affinity chromatography. Lysates from these cells were subjected to SDS-PAGE and Western analysis using antibodies specific for $p27^{Kip}$ and cyclin D2. The lower band in the cyclin D2 doublet represents the faster migrating activated form of cyclin D2. (B) Quantitation was performed by densitometric analysis of bands from day 3.5. The differences between SEB treatment and treatment with IL-10 and SEB were found to be statistically significant for both immunoblots ($P < 001$) by the Student's t-test. Differences were also statistically significant when comparing IL-10 and SEB treatments with those in which IL-2 was also added. $P < .001$ for the $p27^{Kip1}$ blot and $P < 002$ for the activated form in the cyclin D2 blot.

shows the effects of adding IL-2 on the level of cyclins D2. The addition of IL-2 to cells treated with SEB and IL-10 significantly restored the expression of cyclin D2. These data thus demonstrate that one mechanism by which IL-10 inhibits entry into the G_1 phase of the cell cycle is by the blockage of IL-2 production that is critical to the downregulation of $p27^{Kip1}$ and the upregulation of early D-type cyclins in T cell activation. Absence of complete block of $p27^{Kip1}$ induction and restoration of the active form of cyclin D2 as per Figure 8 would suggest other possible IL-2-independent effects of IL-10 on $CD4^+$ T cells.

Reduced Levels of IL-2 Transcription Regulators c-fos and c-jun

To determine the effects of IL-10 on the production of IL-2 at the molecular level, I performed flow cytometry analysis looking specifically at the transcriptional regulatory proteins for IL-2. AP-1 is an important transcriptional regulator for IL-2 and is a heterodimer composed of the proteins c-fos and c-jun (Jain et al., 1992). As seen in Figure 9, flow cytometry showed an increase in the expression of both c-fos and c-jun proteins in $CD4^+$ T cells induced by treatment with SEB, consistent with the cells becoming activated. I further showed that the addition of IL-10 to these cultures along with SEB caused a decrease in the levels of the c-fos and c-jun proteins. There appeared to be a stronger effect of IL-10 on the expression of c-fos than c-jun. These results are consistent with my data showing that IL-10 can significantly decrease the amount of IL-2 produced by cells stimulated with SEB and prevents $CD4^+$ T cells from becoming activated and entering the cell cycle.

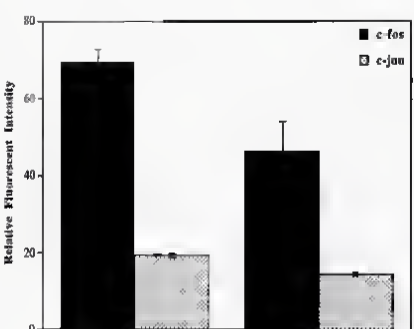

FIGURE 9- IL-IB reduces SEB induction of c-fos and c-jun proteins in CD4⁺ T cells PBMCs were incubated in 6-well plates at 6 x 10^5 cells/ml with media alone, SEB (100 pg/ml), or IL-10 (10 units/ml) and SEB Cells were incubated for 45 minutes, harvested, and subjected to flow cytometry analysis. Data were gated to analyze $CD4^-$ T cells. Results were determined to be statistically significant by the Student's t test ($P< 01$ for c-fos and $P< 0005$ for c-jun) These values represent net effects after the subtraction of relative fluorescent intensity for untreated cells.

IL-10 Directly Blocks Proliferation of $CD4^+$ T Cells

To investigate the effects of IL-10 on $CD4^+$ T cells in the absence of antigen-presenting cells (APCs), I studied the antiproliferative effects of IL-10 on $CD4^+$ T cells in a system depleted of APCs. $CD4^+$ T cells were isolated from PBMCs and their purity was verified by flow cytometry as consisting of 90% $CD4^+$ T cells. These $CD4^+$ T cells were stimulated either with SEB or a mixture of phorbol 12-myristate 13-acetate (PMA) and ionomycin and their proliferation was measured by [^{3}H]thymidine incorporation. SEB is a unique superantigen in that it has been shown to directly activate T cells in absence of APCs (Taub and Rogers, 1992). Figure 10A shows that SEB did induce $CD4^+$ T cells to proliferate in the absence of APCs and that IL-10 was able to significantly block this proliferation. PHA, whose effects are known to be APC dependent (Krutzbeck and Shevach, 1992), had a negligible effect on proliferation of the $CD4^+$ T-cell population used while also stimulating a population that contained APCs. I also stimulated $CD4^+$ T cells with varying mixtures of PMA and ionomycin, which can also directly activate $CD4^+$ T cells. IL-10 significantly inhibited the proliferation of $CD4^+$ T cells at all of the PMA concentrations tested (Figure 10B) and also at various ionomycin concentrations tested (data not shown). These data show that IL-10 had a direct antiproliferative effect on $CD4^+$ T cells and that these antiproliferative effects are independent of IL-10's effects on APCs, such as downregulation of MHC class II molecules and downregulation of CD80/86 molecules. Furthermore, these data provide functional evidence for the presence of IL-10 receptors on $CD4^+$ T cells.

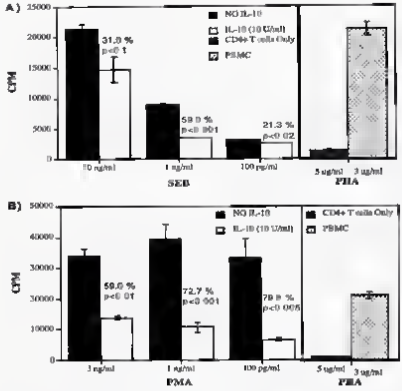

Figure 10: IL-10 blocks proliferation of CD4⁺ T cells: (A) CD4 T cells were isolated from PBMCs by negative selection chromatography and were added to 96-well plates at 2.5 x 10^5 cells/ml along with IL-10 at 10 units/ml or media only. After incubating for 2 hours, SEB at varying concentrations was added and plates were incubated for 4 days. Proliferation was measured by [^{3}H]thymidine incorporation. Data are expressed as the mean CPM ± SD. The percentage of suppression by IL-10 and statistical significance as determined by the Student's t-test, for each treatment are indicated above the graphs. (B) CD4 T cells were incubated as indicated above and stimulated with a mixture of ionomycin (100 ng/ml) and varying concentrations of PMA. Proliferation was measured by [^{3}H]thymidine incorporation. Data are expressed as mean CPM ± SD. The of percentage suppression by IL-10 and statistical significance, as determined by the Student's t-test, for each treatment are indicated above the graphs. In both (A) and (B), the proliferation of CD4 T cells incubated with PHA at 5 ug/ml are compared with that of whole PBMCs incubated with 3 ug/ml

Suppression of Extracellular Signal-Regulated Kinase Activity

Next, the role of inhibition of the MAP kinase cascade by IL-10 was examined. IL-10 has been shown to alter the state of activation of MAP kinases in monocytes (Geng et al., 1994) but the signaling cascades in monocytes differ from those in $CD4^+$ T cells. $CD4^+$ T cells were serum starved to reduce background MAP kinase activity and then treated with IL-10 (100 units/ml) for the times indicated and stimulated as indicated. ERK-1 and ERK 2 were then immunoprecipitated from cell lysates of these cells and the ERK proteins subjected to an ERK kinase assay. As seen in Figures 11 and 12, IL-10 inhibited the ability of the MAP kinase ERK to phosphorylate a substrate as compared to cells stimulated in the absence of IL-10. These data correlate with the previous data indicating that IL-10 acts by suppressing the mechanisms by which the $CD4^+$ T cell produces IL-2.

ERK can also be activated by cell manipulation (Ju et al., 1997) in a time and force dependent manner, explaining the basal level of ERK activity. The presence of IL-10 also downregulates this basal level of ERK activity, as seen in unstimulated cells treated with IL-10 only. These data provide further support for IL-10 suppression of ERK activity.

One of the main substrates of activated ERK in the nucleus is the transcription factor Elk-1 which is phosphorylated by activated ERK (Janknecht et al., 1995, reviewed in Hill and Treisman, 1995). A function of Elk-1 is to act as a transcription factor for c-fos, the member of the AP-1 transcription factor for IL-2. Decreased activation of the MAP kinase cascade by IL-10, resulting in decreased phosphorylation of ERK, would then result in decreased activation of the transcription factor Elk-1. This would, in turn,

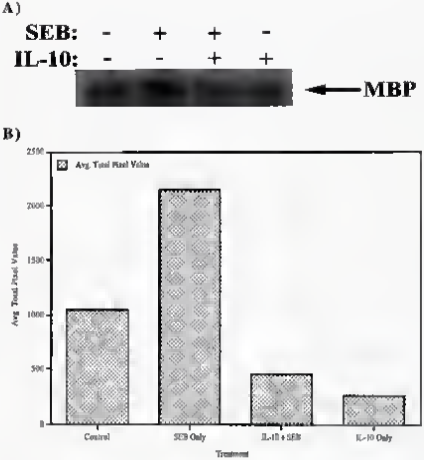

Figure 11. IL-10 Suppresses Erk Activation in SEB-Stimulated $CD4^+$ T Cells. A) $CD4^+$ T cells were incubated in 1.5 ml tubes at 1×10^7 cells/ml along with IL-10 (100 units/ml) or media only for 20 minutes. Cells were then stimulated with SEB at 3 ng/ml or media for 20 minutes, harvested and frozen at -70°C. The cells were then lysed and Erk was immunoprecipitated and subjected to an Erk kinase assay as described using myelin basic protein (MBP) as a substrate. B) Quantitation was performed by densitometric analysis. Results are given for each band as average total pixel value. All differences were found to be statistically significant ($P<.001$) by the Student *t* test.

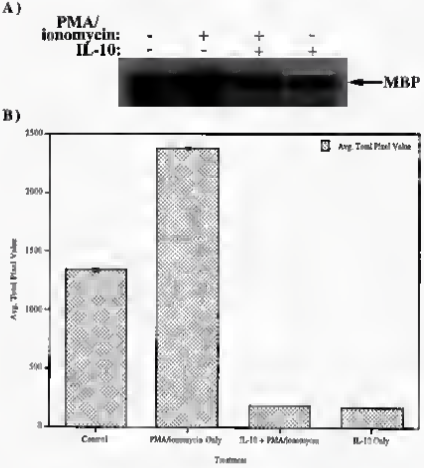

Figure 12: IL-10 Suppresses Erk Activation in PMA/Ionomycin-Stimulated CD4⁺ T Cells. A) CD4⁺ T cells were incubated in 1.5 ml tubes at 1×10^7 cells/ml along with IL-10 (100 units/ml) or media only for 20 minutes. Cells were then stimulated with a mixture of PMA (1 ng/ml) and ionomycin (200 ng/ml) or media for 2 minutes, harvested and frozen at -70°C. The cells were then lysed and Erk was immunoprecipitated and subjected to an Erk kinase assay as described using myelin basic protein (MBP) as a substrate. B) Quantitation was performed by densitometric analysis. Results are given for each band as average total pixel value. All differences were found to be statistically significant ($P < 0.01$) by the Student's t-test.

result in decreased levels of c fos protein in the cells. This correlates with the previous data shown for reduced levels of c-fos in IL-10 treated cells.

Suppression of Raf-1 Activity

In order to further examine the effects IL-10 has on the activation of the MAP kinase cascade proteins the activity of the protein Raf-1 was studied in SEB-stimulated $CD4^+$ T cells. Since Raf 1 exerts its effects upstream of ERK in the MAP kinase cascade, IL-10 effects on Raf-1 may be responsible for the effects seen on ERK. $CD4^+$ T cells were serum starved to reduce basal Raf 1 activity incubated with IL-10 as before and the cells were then stimulated with SEB The frozen cells were lysed as indicated. Raf-1 was immunoprecipitated from the lysates and subjected to a Raf-1 kinase cascade assay Figure 13 shows that IL-10 suppresses the SEB induced activation of the Raf-1 protein. This inactivation of Raf 1 by IL-10 suppresses the entire MAP kinase cascade which helps explain a mechanism by which IL-10 inhibits $CD4^+$ T cell proliferation

The reason for the presence of high levels of Raf-1 activity in cells treated with IL-10 only remains unknown One possible explanation involves the reported association between Raf-1 and the Janus kinase Jak1 (Winston and Hunter, 1995). IL-10 binding to its receptor has been shown to activate Jak1 and tyk2 (Finbloom and Winestock, 1995) It was shown that in cells treated with interferon-γ, Raf-1 could be co-immunoprecipitated with Jak1 (Sakatsume et al , 1998) Furthermore, Jak1 was shown to phosphorylate Raf-1 in this system Since IL-10 induces the activation of Jak1, it is possible that this may allow Jak1 to phosphorylate Raf-1 in a different manner than

normally occurs in the presence of stimulating signals. More research is needed to determine the nature of these interactions.

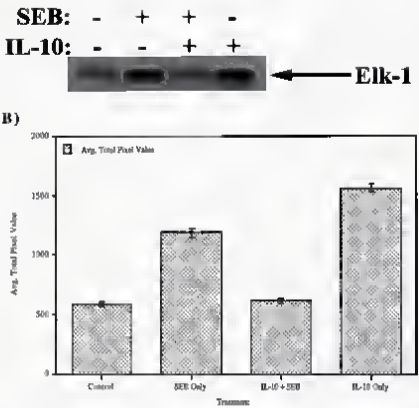

Figure 13: IL-10 Suppresses Raf-1 Activation in SEB-Stimulated CD4⁺ T Cells: A) CD4⁺ T cells were incubated in 1.5 ml tubes at 1 x 10^7 cells/ml along with IL-10 (100 units/ml) or media only for 20 minutes. Cells were then stimulated with SEB at 3 ng/ml or media for 20 minutes, harvested and frozen at -70°C. The cells were then lysed and Raf 1 was immunoprecipitated and subjected to a Raf cascade kinase assay as described using recombinant Elk 1 as a substrate. B) Quantitation was performed by densitometric analysis. Results are given for each band as average total pixel value. All differences were found to be statistically significant (P<.001) by the Student's t-test.

CHAPTER 4
DISCUSSION

The immunosuppressive effects of IL-10 have been well documented in a number of different systems both in vitro and in vivo (Mosmann, 1994). However the mechanism of these effects at the level of cell cycle events is currently not known. Nor is it known how IL-10 exerts its immunosuppressive effects directly on $CD4^+$ T cells. It is also not fully known how IL-10 can inhibit the activation signal from the T cell receptor leading to suppression of IL-2 production in $CD4^+$ T cells. I have initially shown that IL-10 can inhibit the mixed lymphocyte reaction to varying degrees. I have shown also that IL-10 blocks SEB-stimulated $CD4^+$ T cells from entering the cell cycle. Specifically, IL-10 treatment of cells prevented the downregulation of $p27^{Kip1}$, a cell cycle inhibitory protein. Resting cells are characterized by relatively high levels of $p27^{Kip1}$, which binds to cyclin-cdk complexes, inhibiting phosphorylations that are required for moving cells out of G_0 and through the G_1 phase of the cell cycle (Sherr et al. 1994, Koff and Polyak, 1995). IL-10 also prevented upregulation of the G_1 cyclins D2 and D3 in SEB-stimulated $CD4^+$ T cells, which are necessary for the cells to enter the cell cycle. Cyclin D2 has been shown to be present at low levels in resting T cells whereas cyclin D3 is only detected after T-cell activation (Ajchenbaum et al., 1993).

Thus, IL-10 blocked downregulation of $p27^{Kip1}$ and upregulation of cyclins D2 and D3, which provides a mechanism for its inhibition of lymphocyte activation.

Related to the inhibitory effects of IL-10 on $CD4^+$ T cells, IL-10 blocked SEB induction of IL-2. It has previously been shown that IL-10 blocked IL-2 induction, but the consequences of this in terms of cell cycle events have not been determined. Addition of IL-2 to cultures treated with SEB in the presence of IL-10 reversed IL-10 inhibition of $CD4^+$ T-cell cycling. This correlated with downregulation of $p27^{Kip1}$ and upregulation of cyclin D2. Furthermore, because pretreatment with IL-10 did not significantly affect responses to IL-2, it suggests that events affecting the binding of IL-2 to its receptor are probably not involved in the immediate effects of IL-10. However, the reversal by exogenous IL-2 of the effects on the cell cycle proteins $p27^{Kip1}$ and cyclin D2 was not complete. Thus, it appears that IL-10 acts by IL-2-independent mechanisms in addition to suppressing induction of IL-2. I have observed in some instances that IL-2 alone can induce a proliferative response, which varied between donors. However, even in these instances the ability of these cells to respond to IL-2 can be suppressed by IL-10. This would suggest that IL-10 could possibly affect signals that do not directly stem from IL-2 but are nonetheless required in conjunction with IL-2-induced signals in mediating a mitogenic response.

IL-10 was examined for its effect on inhibiting upregulation of the AP-1 transcription factors c-fos and c-jun. These proteins are involved in activation of the IL-2 gene as well as a number of other cytokines involved in cell growth (Jain et al., 1992). Treatment with IL-10 caused a decrease in the amounts of both c-fos and c-jun proteins in SEB stimulated $CD4^+$ T cells. Thus, the effects of IL-10 on c-fos and c-jun could

contribute to the inhibition of IL-2 induction Given the broad gene-target specificity of c-fos and c-jun, it is probable that IL-10 may also have effects that go beyond the early blockage of IL-2 production

By inhibiting the phosphorylation of proteins in the MAP kinase cascade, IL-10 suppresses the activation signal leading from the T cell receptor to the nucleus. This inhibition then, in turn, suppresses the induction of IL-2 production which is necessary for normal T cell activation With IL-2 production inhibited, the cell cycle proteins are maintained in a quiescent G_0 profile characterized by high levels of the cell cycle inhibitory protein $p27^{Kip1}$ and low levels of both cyclin D3 and the activated form of cyclin D2. The $CD4^+$ T cells, therefore, remain in the G_0/G_1 phase of the cell cycle and are inhibited from proliferating normally

Recent studies have shown that IL-10 induced an anergic state in human $CD4^+$ T cells stimulated with alloantigens or cross-linked with anti-CD3 antibodies (Groux et al , 1996, Schwartz, 1996) These studies involved the long-term treatment of cells with IL-10 in which the cells became unresponsive to IL-2 Although I have not directly studied the effect of long-term treatment with IL-10 I have found that pretreatment of cells for up to 2 days with IL-10 did not affect the ability of IL-2 to reverse the cell cycle block. Thus, my data, along with that of others (Groux et al , 1996), would suggest that the effects of IL-10 are two-fold. Short exposures to IL-10 produce an immediate inhibition of the activation of $CD4^+$ T cell entry into the cell cycle. This state of inactivation that is mostly IL-2 dependent and is reflected by high levels of $p27^{Kip1}$, low levels of cyclin D2 and suppression of MAP kinase activity This phase is partially reversible by IL-2. However, continued treatment with IL-10 apparently produces

permanent changes in these cells that ultimately makes them unresponsive to IL-2 and renders the cells anergic. The mechanism of this shift to a permanent state of IL-2 unresponsiveness remains unclear.

The data presented regarding the effects of IL-10 on the activity of proteins of the MAP kinase cascade are also important when considering IL-2-independent effects. The MAP kinase proteins are involved in other cell signaling events besides those coming from the T-cell receptor such as the co-stimulatory signal in the CD28 cascade. There is also evidence that ERK can phosphorylate STAT proteins in the nucleus and that this phosphorylation is required for the full activation of the STAT protein (David et al., 1995). MAP kinases may also cooperate with each other in steroid-dependent transcription via phosphorylation of receptors, as has been shown with the estrogen receptor (Kato et al., 1995). These studies all point to the diverse activities of the proteins of the MAP kinase pathways and suggest that IL-10's effects on these proteins could have a number of diverse outcomes in addition to suppressing the production of IL-2.

IL-10 is produced relatively late in the immune response and is thought to be important in the shift from a cell mediated response to an antibody mediated response. In this regard, IL-10 functions in a paracrine fashion, affecting neighboring cells. Because IL-10 effectively blocked cell cycling in the presence of SEB, our data suggest that IL-10 can prevent naïve $CD4^+$ T cells from being activated even when they are subsequently presented with antigen. This highlights an important use for IL-10 as an immunosuppressive drug. Furthermore, when compared in this fashion, immunosuppressive drugs like cyclosporin A and FK506 would be expected to resemble

IL-10 in their effects on the cell cycle. These drugs act to prevent T-cell activation primarily by blocking IL-2 synthesis (Jain et al., 1992) and, thereby, presumably also preventing the cell cycling of target cells in a manner analogous to IL-10. This is in contrast to drugs like rapamycin that act by blocking signaling following the binding of IL-2 to its receptor (Nourse et al. 1994). Rapamycin, like IL-10, also prevents the downregulation of $p27^{Kip1}$, but this effect cannot be reversed by exogenous IL-2 (Nourse et al., 1994).

Overall, my results suggest the importance of IL-10 in regulating the cell cycle proteins necessary for stimulated $CD4^+$ T cells to enter the cell cycle. They also implicate IL-2 as a key cytokine mediating the early short-term immunosuppressive effects of IL-10 on the cell cycle. I show, for the first time, the ability of IL-10 to inhibit the activation of proteins in the MAP kinase pathway in human $CD4^+$ T cells. These results show both the IL-2 dependent and IL-2 independent mechanisms responsible for the immunosuppressive effects of IL-10 on human $CD4^+$ T cells.

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BIOGRAPHICAL SKETCH

George Q Perrin III was born in Long Island, New York, on December 3, 1963, to Jane and George Perrin Jr. Jane was born in Dublin, Ireland, and grew up one of eight children in the household of Jane and Michael Donohue in the Bronx, New York. George Jr. was the younger of two children and was raised in St. Albans, New York, in the household of Clementine and George Perrin Sr., both of French descent. When George III was six, his family moved to Miramar, Florida, where he and younger sister Ann attended St. Stephen's grade school and Chaminade-Madonna High school. After graduation, George attended Purdue University, where he joined Kappa Delta Rho fraternity and became an avid Boilermaker fan. After a difficult few years, including the death of his father, George left Purdue and moved back to Miramar where he earned an Associate of Arts degree from Miami-Dade Community College while working as a phlebotomist at Hollywood Memorial Hospital. He decided to get his bachelor's degree and moved to Orlando in 1988 to attend the University of Central Florida. In 1990, he earned a Bachelor of Science degree in biology with a minor in chemistry and began working as a laboratory technician at the U.S. Department of Agriculture in Orlando. In 1994, he moved to Gainesville to pursue graduate work at the University of Florida and became a graduate student in the laboratory of Dr. Howard M. Johnson. After graduation, George plans to continue as a postdoctoral fellow in Dr. Johnson's lab. He then hopes to pursue a career in academia and research.

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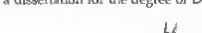

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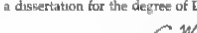

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May 1998

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Dean, Graduate School